UDK 582.282.23.017.7

D.V. Fedorovych[1], M.V. Gonchar[1,3], H.P. Ksheminska[1], T.M. Prokopiv[1], H.I. Nechay[1], P. Kaszycki[2], H. Koloczek[2], A.A. Sibirny[1]

[1]Institute of Cell Biology, NAS of Ukraine, Drahomanov str., 14/16, Lviv, 79005, Ukraine
[2]University of Agriculture in Krakyw, al. 29 Listopada 54, 31—425 Krakyw, Poland
[3]University of Rzeszow, Sokołowska str. 26, 36—100 Kolbuszowa, Poland

MECHANISMS OF CHROMATE DETOXIFICATION IN YEASTS

The main pathway of Cr(VI) (hexavalent chromium) detoxification in microorganisms involves its reduction to Cr(III). Characteristics of chromate reduction in yeasts (isolation, purification and characterization of Cr(III)-biochelated complexes from the culture medium) and the non-enzymatic processes responsible for efficient detoxification of chromate are described. The yeast Saccharomyces cerevisiae was shown to be able to reduce chromate extra-cellularly by the formation of at least two types of stable Cr(III)-biochelated complexes. Selenite-resistant mutants of Phaffia rhodozyma were studied with respect to their ability to reduce Cr(VI). The cells of selenite-resistant mutant sit11 which show strong sensitivity toward Cr(VI), generated an increasing pool of a very toxic radical Cr(V). The role of some extracellular agents such as sulfate and riboflavin in Cr(VI) detoxification in the flavin-overproducing yeast Pichia guilliermondii was also investigated. Our data on modulation of chromate toxicity by riboflavin provide some evidence for the involvement of riboflavin (or its derivatives) in chromate detoxification. The identification of the extracellular chromate-reducing compounds and elucidation of their role in detoxification of chromate will promote the application of the studied yeast cells for Cr(VI) bioremediation and its transformation to Cr(III) bio-complexes with potential pharmaceutical and nutritional importance.

K e y w o r d s: chromate, yeasts, detoxification, Cr(III)-biocomplexes, riboflavin.

Chromium in trace amounts is beneficial to humans, animals, plants and microorganisms; it is an element essential for glucose and fat metabolism, stabilization of the tertiary structure of proteins and nucleic acids [3, 15]. However, at high concentrations it is extremely toxic, mutagenic and carcinogenic, especially in its oxidized hexavalent form Cr(VI). The adverse health effects and diverse cellular and molecular reactions make the research on chromium toxicology and metabolism to be very crucial in terms of both environmental protection and clinical medicine. Parallel studies have been performed at molecular and cellular levels using yeasts, mammalian cells and transgenic mice. However the detailed mechanisms of the cell-chromium interactions are yet to be revealed.

Yeasts which proved to be effective in accumulation of chromium ions and were able to bioconvert them into stable, much less toxic and bioavailable forms, might be applied in the management of chromium-containing wastes, as well as in efforts to produce chromium-enriched biomass, containing biostabilized and nontoxic Cr species for balanced nutrition of mammals and humans [5]. The dual role of chromium in the cell metabolism implies the presence of effective mechanisms for controlling its entry into the cells, accumulation and detoxification. Yeast tolerance to Cr(VI) as well as chromium accumulation inside yeast cells were shown to be dependent on treatment time, metal concentration, biomass density and phase of growth [8, 9, 15].

The main pathway of Cr(VI) detoxification in microorganisms, and particularly in bacteria, involves its reduction to Cr(III) [5, 15]. In yeast, the role of chromate reduction to mitigate the toxicity of Cr(VI) is still open for discussion. It was suggested that the principal reason of the yeast resistance to chromium was a low ability to its absorption [4, 15]. However, for the chromate-resistant strains of *Candida maltosa* a NAD-dependent chromate reducing activity was observed [14]. Recently we have found that some reducing substances, secreted extracellularly by yeast played a significant role in Cr(VI)-detoxification [10, 11]. As a product of chromate reduction, trivalent Cr(III) formed complexes with some specific components of culture liquid which were not adsorbed by the cells. Besides, chromate-resistant mutants of this yeast that exhibited the increased chromate-reducing ability have been selected [2]. At the same time, the rate of chromate reduction did not correlate with chromium accumulation in the cells.

In this article, we present data on chromate reduction in several yeast species including isolation, purification and characterization of Cr(III)-biochelated complexes from the culture liquid. We also describe non-enzymatic processes responsible for efficient detoxification of chromate.

Materials and methods

In this work we have used the following yeast strains: 1) *Pichia guilliermondii* ATCC 201911 (L2) (*MAT-hisX-17*) (*MAT* is a mating type locus, *his* — a locus defining histidine biosynthesis); 2) an industrial strain "Effect" of the yeast *Saccharomyces cerevisiae*; 3) *Phaffia rhodozyma* NRRL Y-10921 as well as its selenite-resistant mutants selected by our team.

The yeast cells were cultivated at 30 °C (for *P. rhodozyma* — at 22 °C) in Erlenmeyer flasks on a circular shaker (200 rpm) in Burkholder's medium with the addition of 0.1% yeast extract (a semi-rich medium) or 0.2% yeast extract +0.2% peptone (a rich medium). Cr(VI) was added to the medium as potassium chromate. Liquid media were inoculated with the cells from the early stationary growth phase in concentration of 5 mg d.w.cells/l. Yeast biomass was determined turbidimetrically at 600 nm (OD_{600}) using gravimetrical calibration. The determination of total chromium content in the cells was performed using either atomic absorption spectrometer AAS-3 (Carl Zeiss, Germany). Cells for analyses were prepared using acid-hydrogen peroxide mineralization as described earlier [7]. The assay of residual Cr(VI) concentration in media was carried out using diphenylcarbazide method [12]. The content of the trivalent chromium was determined by the reaction with Chromazurol S [7]. The presented quantitative data are average values resulting from 2–3 independent experiments. All analytical measurements were performed in 3 duplicates.

Results and discussion

The cells of baker's yeast were shown to be capable of reducing chromate extracellularly and to form at least two types of stable Cr(III)-biochelated complexes. Such Cr(III)-complexes, isolated from a cultural liquid of *S. cerevisiae* industrial strain "Effect" grown for 3 days in the presence of 1 m*M* chromate, were characterized with respect to their molecular weight. Cr(III)—containing compounds were concentrated by freezing-thawing processes and supplied on a column with a molecular sieve Toyopearl HW-40 (80 x 1.4 cm) calibrated by a set of low molecular markers. Two Cr(III)-complexes were isolated differed in molecular weights (M_I = 440±40 and M_{II} = 380±30 Da). For preparative isolation of Cr(III)-biochelates we used ion-exchange chromatography for fractionation of the concentrated extra-cellular liquid from chromate-supplemented culture of *S. cerevisiae*. It was shown that Cr(III)-biochelates could be separated into at least two components on anion-exchanging resin Dowex 1x10 (14 x 2 cm). Both Cr(III)-complexes have a total negative charge. Absorption peaks were observed for Cr(III)-complex I at 572–574 nm and for Cr(III)-complex II – at 579–581 nm (Fig. 1).

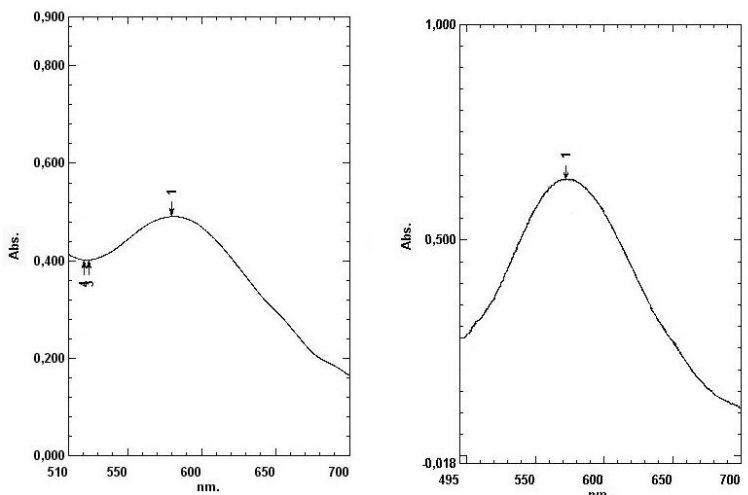

Fig.1. Absorbance spectra of Cr(III)-biocomplexes isolated from a cultural liquid of S. cerevisiae

The selenite-resistant mutants of carotene-synthesizing yeast *P. rhodozyma*, isolated by us previously [13] have been studied regarding their ability to reduce Cr(VI) and to produce astaxantin. The obtained results suggest that although the pathways of detoxification of chromate and selenite by *P. rhodozyma* were different, one common reductive type involved in transformation of these compounds was observed. The EPR spectrometry was employed to follow the reduction of chromate applied to cell cultures. It was shown for the case of the selenite-resistant mutant *sit11* which simultaneously revealed sensitivity to chromate, that this phenotype was accompanied with the increasing pool of the very toxic intermediate, free radical Cr(V) (g=1.98) generated in extra-cellular medium of the culture (Fig. 2). In contrast to the wild-type strain characterized by a relatively constant Cr(V) pool as observed in medium during incubation with chromate, the chromate-sensitive mutant *sit11* (Fig. 2B) generated gradually increasing pool of Cr(V) species.

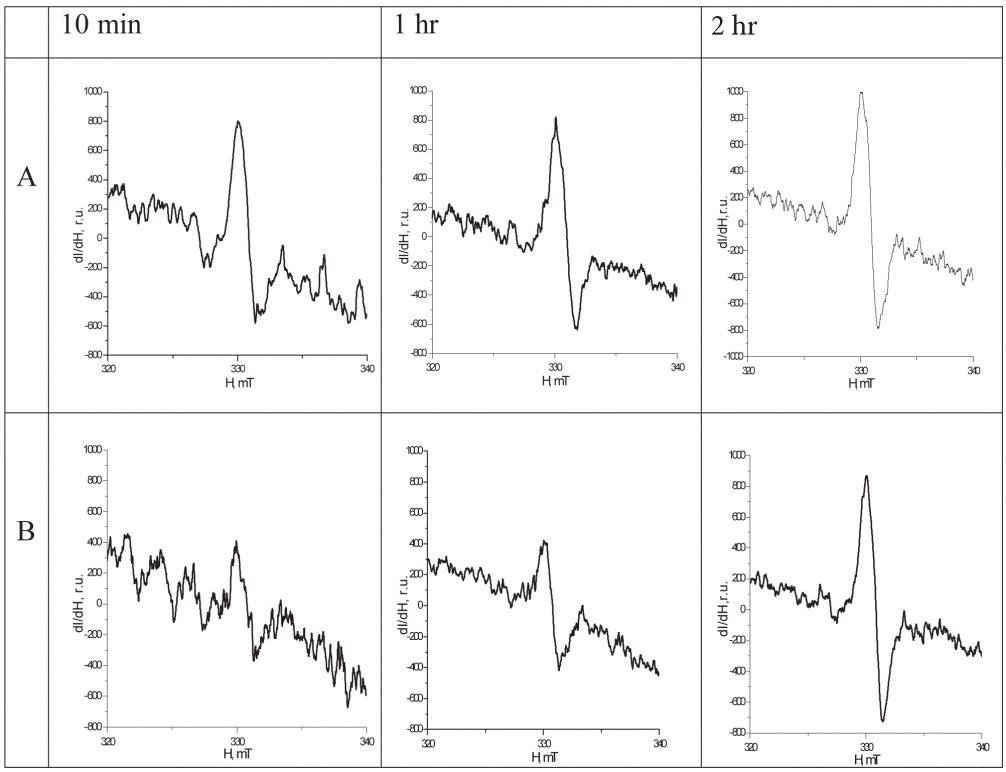

Fig. 2. EPR-spectra of Cr(V) (g=1.98) in cultural liquid of P. rhodozyma incubated with 1 mM chromate (10 min, 1 and 2 hours)

A — parental ("wild type") strain as a control; B — mutant *sit*11. The EPR measurements were performed on the X-band spectrometer RADIOPAN using liquid nitrogen cooling system.

The role of some extracellular agents such as sulfate, iron (II), riboflavin, cysteine in Cr(VI) detoxification in flavin-overproducing yeast *P. guilliermondii* was investigated. The presence of sulfate, a typical media constituent, had a strong negative effect on Cr-bioaccumulation. Under conditions of sulfate deprivation, the cellular chromium content was approximately 20 times higher as compared to the cells grown in sulfur-sufficient media. The flavinogenic yeast *P. guilliermondii* responded to Cr(VI) by stimulation of riboflavin (RF) biosynthesis [6]. We hypothesized that the extensive flavinogenesis was a response to Cr(VI) treatment and that it served as a mechanism leading to higher yeast survival under chromate-induced stress. Nevertheless the sensitivity of the studied yeast strains to Cr(VI) did not correlate with the level of flavinogenic activity (data not shown). However when Cr(VI) was added to the cultures that actively synthesized RF the growth inhibition was suppressed. In order to determine the possible role of exogenous RF in diminishing the inhibitory effect of Cr(III) and Cr(VI) on the culture cell growth, *P. guilliermondii* flavinogenic strains UKD-66 and UKD 1453, unable to oversynthesize of RF in the media supplemented with chromate, were used. The yeast growth in the presence of 200 μg/ml RF was investigated and the cellular content of Cr and flavins was monitored. The incubation of biomass (2 OD_{600}/ml) with chromium and RF was performed during

18 h. In all the cases, the sensitivity of the cells to chromium depended strongly upon presence of exogenous RF. The treatment of the cells with Cr(VI) (0.6 mM) in medium supplemented with RF resulted in an increase of biomass and flavin content (Table), although the addition of RF did not cause any significant changes of cellular Cr content.

It was shown earlier [1] that RF could decrease the nephrotoxic effect of chromate in young and adult rats. Our data on RF protection of *P. guilliermondii* cells against chromium toxicity, for the first time it was provided the evidence on RF involvement in chromium detoxification.

Table

Effect of exogenous RF (200 μg/ml) on the growth, chromium and flavin content in *P. guilliermondii* cells of the strains UKD-66 and 1453 during incubation with chromate (0.6 mM)

Strains	Biomass, mg/ml		Flavins in the cells, μmol/g d.w.		Chromium content in the cells, mg/g d.w.	
	-RF	+RF	-RF	+RF	-RF	+RF
UKD-66	2.16	3.91	43.5	168.3	1.8	1.3
UKD-1453	1.03	3.12	44.2	212.1	1.24	0.8

Identification of the extracellular chromate-reducing compounds and elucidation of their role in detoxification of chromate will promote the application of yeast for chromate bioremediation and its transformation to Cr(III) bio-complexes with potential pharmaceutical and nutritional importance.

This work was supported by the Polish Research Committee grant for the project N N304 326136.

We are thankful to Yurii Usatenko for technical assistance in EPR measurements.

REFERENCES

1. Appenroth D., Shulz O., Winnefeld K. Riboflavin can decrease the nephrotoxic effect of chromate in young and adult rats // Toxicol. Lett. — 1996. — 87. — P. 47–52.

2. Babyak L.Ya., Ksheminskaya G.P., Gonchar M.V., Yanovich D.V., Fedorovich D.V. Selection and properties of mutant yeast *Pichia guilliermondii* resistant to chromium Cr(VI) // Appl. Biochem. Microbiol. — 2005. — 41. — P. 177–181.

3. Cervantes C., Campos-Garcна J., Devars S., Gutiürrez-Corona F., Loza-Tavera H., Torres-Guzmбn JC., Moreno-Sбnchez R. Interactions of chromium with microorganisms and plans // FEMS Microbiol. Rev. — 2001. — 25, № 3. — P. 335–347.

4. Czaky-Vür K., Batiu M., Raspor P., Sipiczki M., Pesti M. Hexavalent chromium uptake by sensitive and tolerant mutants of *Schizosaccharomyces pombe* // FEMS Microbiol. Lett. — 1999. — 178, № 1. — P. 109–115.

5. Demirci A., Pometto A.L. Enhanced organically bound chromium yeast production // J. Agric. Food. Chem. — 2000. — 48. — P. 531–536.

6. Fedorovych D., Kszeminska H., Babjak L., Kaszycki P., Kołoczek H. Hexavalent chromium stimulation of riboflavin synthesis in flavinogenic yeast // BioMetals. — 2001. — 14. — P. 23–31.

7. Honchar T. M., Ksheminska H. P.,Patsay I. O., Huta O. M., Gonchar M. V. Assay of chromium(III) in yeast cultures using Chromazurol S and surfactants for monitoring chromate remediation processes // Biotechnology (Kyiv).— 2008. — 1, № 4. — P. 85—94.

8. Kaszycki P., Fedorovych D., Ksheminska H., Babjak L., Wojcik D., Koloczek H. Chromium accumulation by living yeast at various environmental conditions // Microbiol. Res. — 2004. —159. — P. 11—18.

9. Ksheminska H., Fedorovych D., Babyak L., Yanovych D., Kaszycki P., Koloczek H. Chromium (III) and (VI) tolerance and bioaccumulation in yeast: a survey of cellular chromium content in selected strains of representative genera // Process Biochem. — 2005. — 40. — P. 1565—1572.

10. Ksheminska H. P., Honchar T. M., Gayda G. Z., Gonchar M. V. Extra-cellular chromate-reducing activity of the yeast cultures // Centr. Europ. J. Biol. — 2006. — 1. — P. 137—149.

11. Ksheminska H., Fedorovich D., Honchar T., Ivash M., Gonchar M. Yeast tolerance to chromium depends on extra-cellular chromate reduction and Cr(III)-chelation // Food Technol. Biotechnol. — 2008. — 46. — P. 420—427.

12. *Marchart H.* Reaction of Chromium with Diphenylcarbazide and Diphenylcarbazone // Anal. Chim. Acta. — 1964. — 30. — P. 11—17 (in German).

13. Nechai H. Isolation spontaneous mutants of carotene-synthesizing yeast *Phaffia rhodozyma* which are resistant to sodium selenite // Abs. II Intern. Conference of young scientist "Biology: from molecular to biosphere" (Kharkiv, Nov. 2007). — 2007.— P. 386.

14. Ramнrez-Ramнrez R., Calvo-Mündez C., Avila-Rodrнguez M., Lappe P., Ulloa M., Vбzquez-Juбrez R., Gutiürrez-Corona JF. Cr(VI) reduction in a chromate-resistant strain of *Candida maltosa* isolated from the leather industry // Antonie Van Leeuwenhoek. — 2004. — 85, № 1. — P. 63—68.

15. *Raspor P, Batic M, Jamnik P, Josic D, Milacic R, Pas M, Recek M, Rezic-Dereani V, Skrt M.* The influence of chromium compounds on yeast physiology (a review) // Acta Microbiol. Immunol. Hung. — 2000. — 47. — P. 143—173.

Д.В. Федорович[1], М.В. Гончар[1,3], Г.П. Кшеминская[1], Т.М. Прокопив[1], Г.И. Нечай[1], П. Кашицки[2], Г. Колочек[2], А.А. Сибирный[1]

[1]Институт биологии клетки НАН Украины, ул. Драгоманова 14/16, Львов, 79005, Украина;
[2]Краковский аграрный университет, пр. 29 Листопада 54, 31—425 Краков, Польша;
[3]Жешувский университет, ул. Соколовская 26, 36-100 Кольбушова, Польша

МЕХАНИЗМЫ ДЕТОКСИКАЦИИ ХРОМАТА У ДРОЖЖЕЙ

Реферат

Основным путем детоксикации Cr(VI) у микроорганизмов является его восстановление к Cr(III). Приведены данные о редукции хромата (выделение, очистка и характеристика Cr(III)-биохелатирующих комплексов из культуральной жидкости), а также описаны неферментативные процессы, обеспечивающие эффективную детоксикацию хромата. Дрожжи *Saccharomyces cerevisiae* способны к внеклеточному восстановлению хромата с образованием, по крайней мере, двух типов стабильных Cr(III)-биохелатирующих комплексов. Исследована способность к восстановлению Cr(VI) селенитрезистентных мутантов дрожжей *Phaffia rhodozyma*. Показано, что клетки селенитрезистентного мутанта *sit*11, обладающие повышенной чувствительностью к Cr(VI), при инкубации с хроматом образуют стабильный пул очень токсичного радикала Cr(V). Исследована роль некоторых внеклеточных агентов, в частности сульфата и рибофлавина, в детоксикации Cr(VI) у дрожжей *Pichia*

guilliermondii, способных к сверхсинтезу рибофлавина. Приведенные данные о влиянии рибофлавина на изменение токсичности хромата свидетельствуют об участии рибофлавина (или его производных) в детоксикации хромата. Идентификация внеклеточных хроматредуцирующих веществ и выяснение их роли в детоксикации хромата свидетельствуют о возможности использования дрожжевих клеток для биоремедиации хромата, а также превращении его в Cr(III)-биокомплексы, имеющие фармацевтическое и кормовое значение.

К л ю ч е в ы е с л о в а: хромат, дрожжи, детоксикация, Cr(III)-биокомплексы, рибофлавин.

Д.В. Федорович[1], М.В. Гончар[1,3], Г.П. Кшемінська[1], Т.М. Прокопів[1], Г.І. Нечай[1], П. Кашицкі[2], Г. Колочек[2], А.А. Сибірний[1]

[1]Інститут біології клітини НАН України, вул. Драгоманова 14/16, Львів, 79005, Україна;
[2]Краківський аграрний університет, пр. 29 Листопада 54, 31—425 Краків, Польща;
[3]Жешувський університет, вул. Соколовська 26, 36-100 Кольбушова, Польща

МЕХАНІЗМИ ДЕТОКСИКАЦІЇ ХРОМАТУ У ДРІЖДЖІВ

Реферат

Основним шляхом детоксикації Cr(VI) у мікроорганізмів є його відновлення до Cr(III). Представлено дані з редукції хромату (виділення, очищення і характеристика Cr(III)-біохелатуючих комплексів з культуральної рідини) та описано неферментативні процеси, що забезпечують ефективну детоксикацію хромату. Дріжджі *Saccharomyces cerevisiae* здатні до позаклітинного відновлення хромату з утворенням щонайменше двох типів стабільних Cr(III)-біохелатуючих комплексів. Вивчено здатність селеніт-резистентних мутантів дріжджів *Phaffia rhodozyma* до відновлення Cr(VI). Показано, що клітини селенітрезистентного мутанта *sit*11, які володіють підвищеною чутливістю до Cr(VI), при інкубації з хроматом утворюють стабільний пул дуже токсичного радикалу Cr(V). Досліджено роль деяких позаклітинних агентів, зокрема сульфату і рибофлавіну, у детоксикації Cr(VI) у дріжджів *Pichia guilliermondii*, здатних до надсинтезу рибофлавіну. Наведені дані про вплив рибофлавіну на зміну токсичності хромату свідчать про участь рибофлавіну (або його похідних) у детоксикації хромату. Ідентифікація позаклітинних хроматвідновлювальних сполук та з'ясування їх ролі в детоксикації хромату свідчать про можливість використання клітин дріжджів для біоремедіації хромату та перетворенні його в Cr(III)-біокомплекси, що мають фармацевтичне та кормове значення.

К л ю ч о в і с л о в а: хромат, дріжджі, детоксикація, Cr(III)-біокомплекси, рибофлавін.